# Circadian clock

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treadmill and to be implicated in a mitotic-like process.

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# Circadian Clock: Time for a Phase Shift of Ideas?

**A recent study shows that cycling of cryptochrome proteins is dispensable for circadian clock function in mammalian cells. Is it time for a paradigm shift in how we think about the circadian clock mechanism?**

**Martha Merrow[1,*] and Till Roenneberg[2]**

Clocks rule our lives. One of them is the circadian clock — an adaptive, endogenous temporal programme which ensures that the same processes occur at the same time, day after day. These processes are based on a number of rhythmic functions ranging from gene expression and metabolism to behaviour and they are so pronounced that we are biochemically 'different' people from day to night. Daily rhythms are self-sustained; they even oscillate in the absence of a cycling environment with their period being close to 24 hours, hence circadian. The implications of this circadian programme should be of interest to all biologists and doctors because time of day can potentially impact on all kinds of experiments and medical diagnoses.

The genetic revolution for complex behaviour started with Seymour Benzer who identified the first clock gene, *period,* in *Drosophila* [1]. Since then, clock genes have been described in all circadian model systems, from cyanobacteria to mice. By discovering how the products of these clock genes interact, a cohesive mechanism emerged that is apparently common to all circadian systems: an auto-regulatory negative feedback loop involving transcription and translation (Transcription Translation Feedback Loop, TTFL). Changes in any of the loop's components have dominant effects on the circadian rhythmicity, and it has been hypothesised that this feedback loop generates circadian rhythms at the cellular level (Figure 1A).

In a recent paper in *Current Biology*, Fan *et al.* [2] describe provocative results that — if repeated, extended and elaborated — challenge the current feedback loop model. In mice, levels of the TTFL components mPERIOD (PER) 1 and 2, mCRYPTOCHROME (CRY) 1 and 2, and BMAL1 typically oscillate over the course of a day. The oscillation is thought to depend largely on the CRY proteins, which repress CLK/BMAL1 mediated transcriptional activation. PER proteins also show negative feedback but are less potent than either of the CRY proteins in cell culture systems [3]. Fan *et al.* [2] have engineered the two key negative feedback components, the mCRY proteins, such that they move freely into cells. They surprisingly find that, despite the apparently constant level of both CRYs and CRY-induced BMAL1 expression, Per2 transcription continues to cycle rhythmically. This indicates that one of the fundamental assumptions concerning the mouse circadian clock seems incorrect, namely that oscillations of CRY protein levels

are essential for generating circadian rhythmicity.

There are numerous possible scenarios which could account for the observations of Fan *et al.* [2] from which we have chosen five. Scenario 1: The effects on the molecular machinery of the circadian clock using the new cell-permeable CRY proteins could simply be an artefact of the method itself. Many things happen to clock proteins on their daily journey through time. They are modified, gang up with other proteins, get shuffled between compartments and so on. Does all of this also happen in cells that are flooded with the two CRY proteins? Future experiments will elaborate on the fates of the imported proteins and how they compare with those of endogenous CRY proteins in untreated cells. However, the surprising observation that the constitutively present, cell-permeant CRYs support BMAL1 oscillations in normally arrhythmic Cry1;Cry2 mutant fibroblasts argues against this scenario.

Scenario 2: The traditional feedback loop hypothesis still holds. Experiments in many circadian systems have shown that several interlocking loops form a 'network of time' and the authors may have simply uncovered yet another loop within the network, one that reflects PER2 rhythms versus CRY1 and CRY2 rhythms (Figure 1B). There are already many indications of auxiliary loops involving, for example, nuclear orphan receptors, DECs or PAR-proteins. The findings of Fan *et al.* [2] may be linked to one of these or may lead to a novel extra-TTFL loop.

Scenario 3: The experiment might reveal a similar phenomenon as in *Drosophila*, where constitutive expression of both so-called negative elements of the TTFL–PER and TIM- still allows rhythmicity in locomotor activity as well as in the levels of the clock proteins themselves [4]. The assumption here is that the rhythmicity of the proteins — in terms of level, state or localization — is essential for circadian behaviour, rather than the rhythmicity of their transcription (Figure 1C). Additional support for this idea comes from experiments exposing the clock to different daily environmental cycles in the lab, whereby in the fungus *Neurospora*, it is the clock gene protein levels that correlate with the 'behavioural' phase (in this case, midnight), while the RNA levels appear simply driven (masked) by the changing light levels [5]. In the experiments here, at least PER1 protein levels can oscillate, so perhaps this is the essential rhythmic component, with the rhythms of the other components contributing characteristics of the system that cannot be appreciated in cell culture systems.

Scenario 4: The TTFL is not the core, rhythm-generating mechanism, but rather serves both an input and an output with feedback function. As its input, it processes signals from the environment to the rhythm generator, which in turn regulates the input pathway. This would render the TTFL also an output of the clock and in this role it would transcriptionally control diverse outputs (Figure 1D). Such an alternative hypothesis was initially proposed in 1998 ([6] and references therein) based on mathematical modelling, and since then numerous experimental results in many model systems support this scenario [7]. Light reception and components of the TTFL are tightly associated. In some cases, they serve both functions, in other cases, they have changed their role from one function to the other during evolution [8–10]. Light often leads to rapid changes in the TTFL components, concurring with phase shifts of the circadian oscillation. Others have shown, however, that residual circadian properties persist when components of the TTFL are rendered non-functional. In *Drosophila*, combination of the arrhythmic mutant allele of *per* with $cry^b$ results in the restoration of some circadian properties, rather than an additional decrement [11]. In *Neurospora*, sporadic free running rhythms

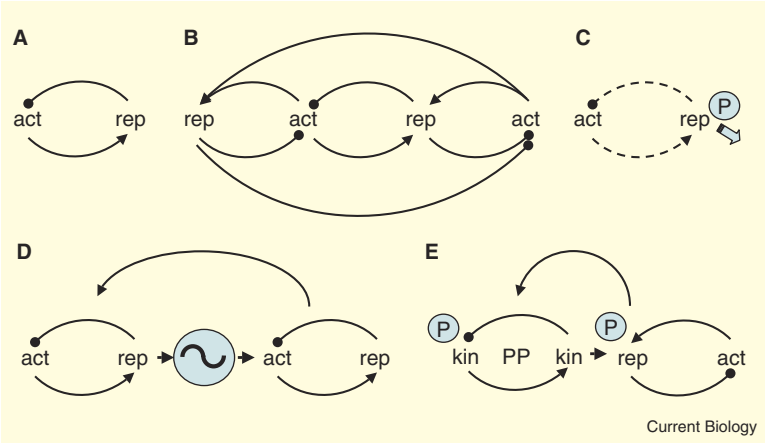


Figure 1. Possible scenarios explaining the dispensability of CRY protein oscillations in the circadian clock.

Five scenarios as described in the text are illustrated. (A) The Transcription Translation Feedback Loop (TTFL) model is built on the elegantly simple hypothesis that clock gene transcription, effected by transcriptional activators (act) is self-regulated by feedback repression (rep). (B) The TTFL network is far more complex, and the actual relationships may be revealed through experiments such as those described in [3]. (C) The role of transcription (dashed lines) may not be critical for all circadian properties. Rather, post-translational modifications, such as phosphorylation (P) or sub-cellular localisation (block arrow) could play major roles. (D) The TTFL components may be serving input and output functions in the clock signal transduction pathway. In this case, the identity of the actual rhythm generator (depicted as a sine wave) may still be unknown. (E) The TTFL may be regulated by a 'phosphorylation oscillator', namely an interaction between a kinase (kin) and a phosphatase (PP) whereby functional activity of clock-critical kinases is modulated via phosphatases, which are in turn regulated by components of the TTFL that are targets of the kinases.

[12,13] and also systematic entrainment characteristics can be found in clock null mutants [14], and subsequently, molecular rhythms have been shown to persist in these strains [15]. In mice, many mutants that had been considered 'clock null mutants' because they were arrhythmic (e.g., the mPer2;mCry2 double mutant) become rhythmic when put into constant dim light [16]. Furthermore, conceptual models show that alterations of input feedback components can have huge effects on circadian oscillations [6]. Finally, feedbacks of the rhythm generator back onto its inputs have been shown to shield the rhythm from noise by compensation and, thus, add robustness to the endogenous daily cycle [6].

Scenario 5: Some of the scenarios above indicate that transcription and/or translation may not need to be rhythmic to ensure circadian function. But where, then, is rhythmicity generated? A recent finding in cyanobacteria suggests that phosphorylation plays a crucial role. Phosphorylation not only confers specific half-lives to proteins, it also regulates their sub-cellular localization and their switching between activity or inactivity — not only in transcription factors, which are so much at the heart of the traditional hypothesis of rhythm generation. In cyanobacteria, three proteins and ATP were shown to be sufficient recapitulate most of the circadian clock properties *in vitro* [17]! Given the added complexity in eukaryotes, we may not expect to find a similar, simple molecular clock mechanism. However, there may be elements of a phosphorylation oscillator [18] that are preserved and remain to be described, but such a mechanism could have also co-evolved by recruiting any of the many autocatalytic kinases and phosphatases present in different species. An indication that a phosphorylation oscillator might be a primordial circadian mechanism comes from several independent observations concerning human kinases and phosphatases. Human CKI autoregulates according to phosphorylation state and is dephosphorylated by PP5 ([18] and references therein), thus these two components could form a feedback loop if they display the correct kinetics and if they were connected to an input pathway. PP5 activity, in turn, depends on binding to CRY2 ([18] and references therein), which fluctuates in concentration in many cells. Even without oscillations in the amount of CRY2, rhythms in the state of phosphorylation could play a part in a phosphorylation oscillator mechanism and might explain the results shown with the cell-permeable CRY1 and CRY2 by Fan *et al.* [2].

Why is it important to understand how the circadian system is put together? Several reports have demonstrated links between health and behaviour with respect to chronotype. A recent publication showed that the erectile dysfunction drug sildenafil has a profound impact on re-synchronization of the circadian system in hamsters [19]. If we are to start medicating the circadian system itself or use a robust circadian system to medicate other pathologies, we have to know what we're aiming for and how the parts of the system are put together.

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